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A CONTRIBUTION TO OUR KNOWLEDGE OF THE RELATION OF CERTAIN SPECIES OF GRASS-GREEN ALGÆ TO ELEMENTARY NITROGEN

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A general survey of the literature pertaining to the relation of algæ to free atmospheric nitrogen reveals the fact that comparatively few forms have been experimented with under conditions which render the conclusions reached free from objection. The principal fault which may be found with most of the work done is that the experiments were carried out with impure cultures. Representatives from not more than four or five genera of green algæ have thus far been studied in pure culture, and while the general conclusion reached is that these forms are unable to fix free atmospheric nitrogen either in the presence or in the absence of combined nitrogen and energy-furnishing materials, it is by no means certain that forms do not exist which, under one or all of these conditions, are able to utilize elementary nitrogen. This thought is especially justified when the small number of free-nitrogen-fixing species among the bacteria is considered. In the present investigation, therefore, an attempt has been made to extend the observations over a greater variety of forms in pure culture,—understanding by the latter a single species of alga free from all other organisms.

HISTORICAL

As early as 1854 Laurent (20, 21), and Morren (24) occupied themselves indirectly with the relation of algæ to free atmospheric nitrogen. Morren was led to the conclusion that the sudden death of cultures of infusoria and algæ was due to the insufficient quantity of combined nitrogen furnished when the number of organisms became considerable. The nitrogen requirement, he found, could be satisfied by ammonium carbonate, organic nitrogenous compounds (decaying insects), and other nitrogenous substances in the water; but in no case did he find that free nitrogen from the atmosphere could serve as the source of nitrogen. While it is difficult to say with what organisms Morren worked, it is altogether probable that members of the *Volvocaceæ* were present among his "green," "brown," and "red infusoria."

No additional contribution to the subject, so far as the author is aware, was made until the appearance of Frank's paper (9) in 1888. In his investigation of the question of a possible fixation of free atmospheric nitrogen in natural soil without the instrumentality of cultivated plants, Frank exposed samples of unsterilized soil, poor in organic matter, in containers under a glass roof, watering them only with distilled water. During the 134 days that the experiment was continued, no phanerogams appeared, but in all cases the surfaces of the soil samples became covered with a thin, crustlike, greenish layer composed of "zwei spangrüne Oscillariaformen, die eine dick-, die andere sehr dünnfädig; ferner grünes Chlorococcum humicola, vielleicht auch Pleurococcus, sowie Vorkeimfäden von Moosen, also kryptogame Gewächse . . . Diatomaceen waren nicht zu finden." Analysis showed an undoubted increase in total nitrogen in the experiments. No increase in the nitrate content was observed,—the additional nitrogen being wholly in the form of organic nitrogenous compounds. These facts led the author to the conclusion that the abundance of algal cells, which are rich in protoplasm and therefore in organic nitrogen, accounts for the presence of the increased nitrogen in an organic form. That the appearance of the nitrogen in an organic form (algal substance) does not represent the primary fixation of free nitrogen and that the latter depends on an inorganic process, the inorganic compounds thus produced being subsequently assimilated by the algæ, is not rendered probable by later experiments. In these, Frank exposed samples of soil, kept free from vegetation, for long periods of time and at various temperatures. Plant growth was prevented by leaching the samples daily with hot water. In this manner any traces of nitrogen compounds formed were also obtained. Only at high temperatures—too high for plant growth—did he find a slight increase in total nitrogen and therefore believes that this process is of no importance under conditions which admit of plant growth. From these observations Frank concludes that the algæ themselves are the immediate agents in the fixation of free atmospheric nitrogen and inclines to extend this faculty to green plants in general.

In the same year, Gautier and Drouin (11) ascribed an entirely different function to soil algæ. Samples of artificial soils, free from organic material and containing only ammoniacal nitrogen, were exposed in a sheltered position for a considerable period of time. During the progress of the experiments the soil became more or less covered with a layer of green algæ (*Pleurococcus vulgaris, Protococcus viridis,* etc.). Analysis showed, in every case, a loss in total nitrogen, an even greater loss in ammoniacal nitrogen, and an intermediate gain in organic nitrogen. The authors assumed that the nitrogen lost was in the form of ammonia and that the amount of nitrogen appearing in the organic form was that part of the escaping ammoniacal nitrogen which, in bathing, so to speak, the algal cells on the surface, was absorbed, and subsequently built into organic nitrogen compounds. In support of this hypothesis the authors state that in proportion to the intensity of the algal growth loss in total nitrogen was diminished, and the amount of ammoniacal nitrogen converted into organic nitrogen increased. Gautier and Drouin thus looked upon the algæ as fixers of gaseous ammonia, which the soil tends to give off constantly, rather than as direct agents in the fixation of free atmospheric nitrogen.

In 1889, Frank (10) made the fixation of elementary nitrogen by soil-inhabiting algæ the subject of a special investigation. Four flasks containing sand moistened with distilled water and plugged with cotton were treated as follows: Two were at once placed in the light; the third was covered with black paper and without further treatment placed with the first two; the fourth was exposed for six hours to a temperature of 100°C. and then placed with the rest. In the first two, rich algal growths developed, composed of two species of *Oscillatoria,* a blue-green "*Nostoc*-Form," a yellowish green "*Nostoc*-Form," a yellowish to pure green *Microcystis,* and a *Glœocapsa.* In the third and fourth flasks no growth of any kind developed. Analyses demonstrated that the total nitrogen content in the first two flasks had been doubled, whereas that in the latter two had suffered a distinct loss. The experiments were repeated with unsterilized soil, all air gaining access to the flasks being first passed through sulphuric acid to remove any ammonia present. The same characteristic algal flora developed and analysis again showed a decided increase in total nitrogen. On the basis of these experiments, Frank makes the generalization that the soil, as such, is unable to fix free atmospheric nitrogen, and that when the process does take place, it is effected by means of the vegetation of low algæ which develop in the soil, and which possess the ability of assimilating free gaseous nitrogen into vegetable, nitrogen-containing compounds. He goes still farther and states that the fact that low algæ utilize free nitrogen makes it more and more probable that the assimilation of elementary nitrogen is a faculty appertaining to the entire plant world provided with chlorophyll, and that, since the simple algal cell is endowed with this faculty, the thought is justified that the assimilation of free atmospheric nitrogen is as absolute and fundamental a process of the entire plant kingdom as is the assimilation of carbon dioxide.

Prantl (27), in cultivating fern prothallia in solutions with and without combined nitrogen, observed that whereas an abundant algal vegetation appeared in the former, only an *Anabœna,* or a *Nostoc,* grew in the latter. When placed in nitrogen-free media, the blue-green alga always grew abundantly. From this observation, and without analytical data, Prantl assumes that free-nitrogen assimilation had taken place, either a direct one by the alga, or an indirect one in which the alga assimilated the ammonium nitrite which, according to the theory of Schoenbein, is formed in the vaporization of water. Of interest are the observations by the same author on the unicellular grass-green algæ, which he was unable to cultivate in solutions free from combined nitrogen. To these, therefore, he assigned the power of elementary-nitrogen fixation in a much smaller degree than to *Nostoc.*

Frank's conclusions were confirmed by the work of Schloesing and Laurent (31). These investigators supplemented the usual indirect method of analyzing the soil and harvest, with the direct method of determining at the beginning and at the end of the experiment the composition of the atmosphere in which the plants had been growing. To 2000 or 2500-gram quantities of a poor sandy soil 2.5 grams of limestone, 5 grams of a mixture of several rich soils, and a certain volume of a mineral nutrient solution containing, in some cases, a little potassium nitrate were added, and the whole placed in large flasks. In some, seeds of Jerusalem artichoke, oats, peas, and tobacco were planted; others, to be used as checks, remained unplanted. To each flask were added 5 cc. of a liquid obtained by diluting 5 grams of rich soil with 20 cc. of water. After fourteen weeks, during which time the seeds germinated and produced plants, the direct analytical method, confirmed by the results obtained by the indirect method, showed, except in two checks, an absorption of free atmospheric nitrogen. But the surfaces of the soils, during the progress of the experiments, became covered with green, cryptogamic plants, among which were mosses (*Bryum*, *Leptobryum*), and algæ (*Conferva*, *Oscillatoria*, *Nitzschia*). This fact led the authors to repeat the first series of experiments, in every case suppressing the growth of chlorophyllous cryptogams by covering the soils with a thin layer of dry, calcined, quartz sand. No trace of algæ or mosses appeared, and, except in the case of the peas, no absorption of free atmospheric nitrogen was observed. This fact, together with the evident fixation of nitrogen in the checks of the first series (in which an abundant chlorophyllous cryptogamic vegetation but no phanerogamic vegetation developed), and the absence of fixation in those checks in which little or no algal growth developed, led Schloesing and Laurent to conclude that there are some "inferior green plants" which are able to utilize free atmospheric nitrogen. In the same year, Gautier and Drouin (12) reasserted their former conclusion as to the rôle of algæ in nitrogen fixation, holding that the methods of those who adhere to the opinion that algæ fix free nitrogen are too faulty to make conclusions drawn from them convincing.

In the work reported by Schloesing and Laurent in 1892 (32, 33) an attempt was made to reduce the complexity of the algal cultures by introducing into a single experiment only one or at most a few species of the algæ. All cultures were made on 600-gram quantities of either a subsoil or quartz sand to which was added (except in the two checks) a small quantity of an infusion prepared from soils. The cultures were allowed to develop for from three to six months, and, as in the previous experiments of these authors, analyses were made both of the contained atmosphere and of the soil and algal growth. The chlorophyllous plants which appeared in the various cultures are described as follows: I and II—essentially a mixture of *Nostoc punctiforme* Hariot and *Nostoc minutum* Desmazières, with a few colonies of *Cylindrospermum majus* Kuetz.; III—almost a pure culture of *Nostoc punctiforme;* IV—*Nostoc punctiforme* (less pure than in III), one colony of *Phormidium papyraceum,* and a small quantity of *Nostoc minutum;* V—two mosses—*Brachythecium rutabulum* and *Barbula muralis;* VI—an almost pure culture of an *Oscillarieæ* and *Microcoleus vaginatus,* with traces of *Tetraspora, Protococcus, Stichococcus, Ulothrix,* and *Lyngbya;* VII and VIII—checks with no growths, or at most a few small patches of *Phormidium autumnale* Gomont and *Nostoc punctiforme.* Both analytical methods showed abundant nitrogen fixation in the first four cultures but not an appreciable one in the fifth,—a fact which the authors explain on the basis of specific differences in plants in their ability to fix free atmospheric nitrogen. The checks showed no appreciable fixation. Separate analyses were made of the top-soil layers, containing the algal growths, and the deeper layers, the increased nitrogen being found in the algal stratum,—a fact which the authors consider important in proving that the algæ were responsible for the free-nitrogen fixation. In conclusion, Schloesing and Laurent admit the possibility that the bacteria present in the cultures had something to do with the fixation of free nitrogen, and state that it is not possible to affirm with certainty that the algæ, free from other organisms, are able to effect fixation. Having observed, however, but few bacteria in the cultures they conclude that the algæ after all are the active agents in the fixation of elementary nitrogen.

Similar results were obtained by Koch and Kossowitsch (17). Sixty grams of washed, calcined sand were placed in large Erlenmeyer flasks and moistened with a mineral nutrient solution free from combined nitrogen. Since previous experiments had shown that algæ do not grow on sand free from combined nitrogen, 0.04 gram of calcium nitrate dissolved in 50 cc. of water were added to each flask. After inoculation with a suspension of algal cells obtained from heaps of lime, a continuous slow stream of air, washed in sulphuric acid, was passed through all the flasks. Three cultures were placed in a north window, three in the dark (to determine whether the bacteria contained in the cultures fixed free nitrogen), and the remainder were used in determining the initial total nitrogen. After fifteen weeks, during which time a rich algal vegetation[1] developed on all cultures exposed to the light, the contents of the flasks were analyzed *in toto*. Those exposed to the light showed an undoubted increase in total nitrogen, whereas those in the dark showed a slight loss in each case. Of particular interest was one culture which was brought into the light after it had remained in the dark for a considerable length of time. After the removal, a moderate growth of algæ appeared, and analysis showed a slight gain in total nitrogen, which, however, was less than that found in the cultures which had been exposed to the light during the entire period. In agreement with the earlier workers, these authors ascribed to algæ the faculty of free-nitrogen fixation, and emphasized the observation that the extent of this fixation was directly proportional to the intensity of the algal development. Petermann (26) reached a similar conclusion on the basis of experiments conducted on sterilized and unsterilized soils, which were respectively inoculated and uninoculated with algæ. The former in each case showed a distinct gain in nitrogen, whereas the latter showed either no increase or a slight loss.

[1] The authors failed to state what algæ developed, merely mentioning the presence of green and blue-green forms.

Incidental to his work on the respiratory quotient in algæ, Schloesing (30) reported that in a culture containing principally *Protococcus vulgaris* Ag., and smaller quantities of *Chlorococcum infusionum* Menegh., *Ulothrix subtilis* Kütz., and *Scenedesmus quadricauda* Bréb., there was at the end of two months no diminution of nitrogen in the supernatant atmosphere. This fact led the author to place these algæ among those forms which do not fix free atmospheric nitrogen.

As will have been observed, the work reported upon in the contributions cited was done with impure cultures. While in some cases but a single species was used, bacteria were present in all cases. Although in many instances this is not expressly stated, the author's experience convinces him that the technique employed by these earlier workers made the contamination of their cultures with bacteria very probable. It is evident, therefore, that in the work done thus far it is impossible to state with certainty whether the results obtained are due to the activity of the algæ, or to the bacteria, or to both.

The first work done on the fixation of free nitrogen by algæ in which pure cultures were used was that of Kossowitsch (18), in 1894. The only form isolated in pure culture by this investigator was one which he states resembled both *Cystococcus* (Nägeli) and *Chlorella vulgaris* Bey. He leaves its identity uncertain but designates it, for convenience, *Cystococcus*. Preliminary experiments with impure cultures of this alga had demonstrated that asparagin and ammonium tartrate could not serve as the source of nitrogen and that growth took place only when nitrates were supplied. In the experiments with pure cultures, flasks containing 70 grams of clean sand moistened with a mineral nutrient solution containing a known amount of calcium nitrate were inoculated with a carefully tested pure culture of *Cystococcus* and allowed to remain four months. To a number of the cultures dextrose was added, and to others, in addition to this sugar, pea-tubercle bacteria. At the conclusion of the experiments the cultures were carefully tested for purity. Analysis in every case showed an absence of free-nitrogen fixation, and demonstrated clearly for the first time that an alga, *Cystococcus*, under the conditions realized in the experiment, did not fix free atmospheric nitrogen. That the same holds true for this alga in nature seemed probable to Kossowitsch, who found that it grew vigorously only so long as a nitrate was present. He further observed that after growth had ceased in any culture, it was promptly resumed upon the addition of a nitrate solution, but not when the nitrogen-free nutrient solution was added. Similar cultures were started in which the inoculation material was either a mixture of algæ and bacteria derived from soil or lime, or a mixture of soil bacteria with a pure culture of *Cystococcus*. In each case the cultures were set up with and without dextrose. Table I gives the results of these experiments.

TABLE I

RESULTS OF KOSSOWITSCH'S EXPERIMENTS WITH PURE AND MIXED CULTURES

+ or − Sugar	Content of cultures	Mg. of N in cultures	
		Initial	Final
− +	*Cystococcus* (pure culture)	2.6 2.6	2.7 2.7
− +	*Cystococcus, Phormidium,* soil bacteria, moulds	2.6 2.6	7.1 9.5
− +	Pure *Cystococcus* culture and bacteria	2.6 2.6	3.1 8.1
− +	*Stichococcus* and bacteria	2.6 2.6	2.3 2.7
− +	*Nostoc,* large round alga, *Scenedesmus,* soil bacteria	2.6 2.6	? 19.1
− +	*Nostoc,* a *Cylindrospermum* (small form), soil bacteria	2.6 2.6	8.8 25.4

Cystococcus, in pure culture, was again unable to fix free gaseous nitrogen, and the same conclusion is reached by Kossowitsch for *Stichococcus,* which even in the presence of a mixture of bacteria failed to fix elementary nitrogen. Of especial interest are the cultures of pure *Cystococcus* with bacteria, as in these the fixation is ascribable only to the bacteria. Which of the organisms in the remaining cultures are responsible for the marked fixation of free atmospheric nitrogen it is impossible to say, the author states. However, from his own results, and those of previous investigators, that the presence of algæ exercises a favorable effect on the process of free-nitrogen fixation, and, further, that the algæ thus far studied in pure culture do not possess this faculty of fixation, Kossowitsch concludes that the algæ play an indirect rôle. He believes they do this by furnishing, through their photosynthetic activity, carbohydrates to the nitrogen-assimilating bacteria. He would look upon the algæ as occupying the same position with reference to free-living, nitrogen-fixing bacteria as the legumes do with reference to the nodule organisms.

Stocklasa (35), while not making his conclusion very clear, leads one to believe that he considers certain algæ (which he fails to enumerate) capable of fixing free atmospheric nitrogen. Unfortunately, all of Stocklasa's experiments were carried out with impure cultures. Molisch (23), in conducting experiments with algæ relative to the necessary nutrient elements, attempted to cultivate *Microthamnion Kützingianum* Näg., *Stichococcus bacillaris* Näg., *S. major* Rbh., *Ulothrix subtilis* (?) Kütz., and *Protococcus sp.*—all in impure culture—on a nitrogen-free mineral nutrient solution. In every case the algæ failed to grow, and Molisch was led to the conclusion that algæ require combined nitrogen for their development. Although no experiments in which combined nitrogen was furnished to the algæ were conducted, the author nevertheless makes the statement, based principally on the work of Kossowitsch just reviewed, that algæ are not able to fix free atmospheric nitrogen.

In the next year Bouilhac (4) reported that he had succeeded in isolating in pure culture *Schizothrix lardacea, Ulothrix flaccida,* and *Nostoc punctiforme.* Unfortunately, this author does not give a detailed account of his isolation methods. Six flasks containing a mineral nutrient solution free from combined nitrogen were inoculated with each alga, and to three of each a drop of soil suspension was added. No growth whatever developed in any of the *Schizothrix* and *Ulothrix* cultures, nor in the *Nostoc* cultures to which the suspension had not been added. But in those cultures of the latter to which a drop of soil suspension had been added, a splendid growth appeared and in each culture analysis showed a nitrogen fixation of from 11 to 23 milligrams. From a second series (in which the cultural solution contained per liter 0.1 gram arsenic acid in the form of potassium arsenate) a similar result was obtained, with fixation of nitrogen of from 5 to 60 milligrams. The presence of *Ulothrix* or *Pleurococcus* in addition to the *Nostoc* and bacteria seemed to have no appreciable effect on the quantity of nitrogen fixed. Bouilhac thus concluded that *Schizothrix lardacea* and *Ulothrix flaccida* (either alone or in the presence of soil bacteria) and *Nostoc punctiforme* (in the pure state) are unable to fix free atmospheric nitrogen in the absence of combined nitrogen. The abundant fixation in the cultures containing a mixture of *Nostoc* and soil bacteria is not ascribed by the author to the activity of either organism alone.

Richter (28) observed pots of soil with and without plants, some placed in the dark, others in the light. While a rich algal vegetation developed in the latter, none appeared in the former. Only in a few cases was the growth accompanied by a marked free-nitrogen fixation, but in these instances the author believes it due to the algæ. Pure cultures were not employed. Benecke (1) contributed some observations made on cultures of *Hormidium, Vaucheria, Cladophora,* and members of the *Conjugales,*—all containing bacteria. In nitrogen-free cultures there appeared what Benecke termed "nitrogen-hunger," a condition which is characterized in *Hormidium* by the production of very long, pale filaments, the cells of which become extremely long and in which the development of the chloroplast is so meager that the cells are almost colorless. Stocklasa (36) found that the "Alinit" bacteria fix free gaseous nitrogen in much larger quantities when grown in the presence of species of *Stichococcus* and *Nostoc.* This influence he considers to be due to the pentosans which, according to his belief, are present in large quantities in various algæ, and which, because of their ready solubility in water, serve as a favorable energy-furnishing medium for free-nitrogen-fixing bacteria.

A noteworthy contribution to the subject is that of Krüger and Schneidewind (19). These authors for the first time conducted extensive experiments with a variety of algæ in pure culture, including *Stichococcus chloranthus, S. major, S. bacil-*

laris, and *S. sp.*, the latter isolated from five different sources; *Chlorella sp.*, from the group of which *Chlorella vulgaris* Bey. is typical (also isolated from five different localities); *Chlorella protothecoides* and three other isolations of a form or forms belonging to the same group; *Chlorothecium saccharophilum* and five other isolations of forms belonging to the same group; and lastly, *Cystococcus humicola.* The media employed by the authors included the following:

1. One per cent dextrose, 0.2 per cent K_3PO_4, 0.04 per cent MgSO4, 0.02 per cent $CaCl_2$, and 1 drop of a 2 per cent $FeCl_3$ solution to each 100 cc. of solution.
2. Ignited sand moistened with solution 1.
3. Solution 1 plus 0.25 per cent $(NH_4)_2SO_4$, and 0.25 per cent $NaNO_3$.
4. Ignited sand moistened with solution 3.
5. One-half per cent beef extract, $\frac{1}{2}$ per cent peptone, and $\frac{1}{2}$ per cent dextrose.
6. Ignited sand moistened with solution 5.
7. Diluted beerwort.
8. Ignited sand moistened with solution 7.
9. Humoüs clay soil plus 35 per cent sand moistened with distilled water.

The results obtained were uniform in that the media, free from combined nitrogen, failed to produce a healthy growth, whereas those containing nitrogen in a combined form showed an abundant growth,—some of the algæ preferring the nitrogen in an organic and others in an inorganic form. Further, no fixation of free atmospheric nitrogen was noted in any of the cultures. Krüger and Schneidewind conclude that there is a strong probability that all other chlorophyllous soil algæ of this kind are unable to fix free atmospheric nitrogen, and, in general, agree with the opinion of Kossowitsch that the soil-inhabiting algæ supply the free-living, nitrogen-fixing organisms with the necessary non-nitrogenous, energy-furnishing material.

Conclusions similar to those of Kossowitsch were reached by Deherain and Demoussy (8), who succeeded in cultivating blue lupines free from root nodules in humus-free sand, the surface of which became covered with *Phormidium autumnale* and *Ulothrix flaccida* in the course of the experiments. The authors explained the growth of the lupines by supposing that the soil bacteria fixed free nitrogen at the expense of energy-furnishing organic materials supplied by the algæ, and that the nitrogen so fixed in organic form became available to the legumes.

A return to the conclusion that members of the *Cyanophyceæ* fix free atmospheric nitrogen is found in an investigation by Beyerinck (2). From 1½ to 2-liter portions of tap or distilled water containing 0.02 per cent dipotassium acid phosphate were inoculated with 1–2 grams of garden soil, and placed in the light. After several weeks a characteristic growth of blue-green algæ developed, containing, among other species, *Anabæna catenula*, a form related to or identical with *Nostoc paludosum*, and *Nostoc sphæricum*,—all non-motile species of *Cyanophyceæ*. The development of the blue-green algæ in an almost nitrogen-free medium led Beyerinck, without analytical data, and in spite of the evident contamination of his cultures with soil bacteria, to the conclusion that the *Cyanophyceæ* belong to the class of organisms possessing the faculty of free-nitrogen fixation. He regards the *Cyanophyceæ* as the only known organisms capable of synthesizing their organic materials from carbon dioxide and free nitrogen, and considers as significant in this connection the observations of Graebner (13) and Treub (37), who found that in the sequence of floras on fresh sand and lava soils, species of *Cyanophyceæ* are the first to appear.

Cystococcus humicola was once more subjected to a careful investigation by Charpentier (7). His previous experiments had demonstrated that the dry weight of algal growth obtained in liquid glucose media was about one-half that of the weight of glucose consumed, and that 5.14 per cent of this dry weight was nitrogen. He then pointed out that the quantity of nitrogen furnished by Kossowitsch to his pure cultures of *Cystococcus humicola* in the form of potassium nitrate was sufficient to produce at least 40 milligrams of growth (dry weight), and that while this growth was being produced it might not be necessary for the alga to seek nitrogen from the atmosphere. Once the dextrose was exhausted, the alga might, it is true, develop at the expense of atmospheric carbon dioxide, but the author holds the opinion that this would mean a double expenditure of energy for the assimilation of both carbon dioxide and free nitrogen and that under these conditions growth would be difficult. Because of the vast amount of energy necessary for free-nitrogen fixation, as illustrated by *Clostridium Pasteurianum*, the author suggests that there is a strong probability that *Cystococcus* is capable of assimilating free nitrogen only when the expenditure of energy in carbon assimilation is reduced to a minimum,—that is to say, when abundant available organic materials are furnished. He further emphasizes the necessity of employing combined nitrogen in a less readily available form than nitrates, suggesting organic nitrogenous compounds. On media composed of a decoction of beans to which were added 1 per cent and 2 per cent of dextrose and gelatin, respectively, *Cystococcus* was grown and the entire culture analyzed for total nitrogen. Although care was taken to have an abundance of available organic material (dextrose) present, Charpentier found that in no case was there any indication of free-nitrogen fixation. He further found that ammonia, asparagin, and peptone were each able to serve as the sole source of nitrogen.

The association of blue-green algæ and soil bacteria is again referred to as an effective agent in free-nitrogen fixation by Bouilhac and Giustiniani (5, 6). Buckwheat, white mustard, corn, and cress were planted in clean sand moistened with a mineral nutrient solution free from combined nitrogen, and the substrata inoculated with *Nostoc punctiforme* and *Anabæna sp.* covered with bacteria. The phanerogams grew to maturity and analysis showed a marked fixation of free atmospheric nitrogen.

Of particular interest are the observations of Heinze (14), who, however, fails to state whether or not the *Chlorella* experimented with was in pure culture. He found that no appreciable growth took place in cultural solutions free from combined nitrogen, but that in the presence of the latter a rich growth appeared, unaccompanied, however, by a definite fixation of nitrogen. More important are his experiments with *Nostoc* in impure condition, a good growth of the form being obtained in a mineral nutrient solution free from combined nitrogen and sugar. These cultures, as well as others on soil inoculated with a similar *Nostoc* culture contaminated with bacteria and fungi, showed a definite amount of free-nitrogen fixation. Heinze was unable to find *Azotobacter* present, and this, together with the observation that the contaminating fungus in pure culture was unable to fix free nitrogen, led the author to the conclusion that the *Nostoc* is, in all probability, directly responsible for the free-nitrogen fixation. Further, he would place *Azotobacter* in close relationship with the *Chroococcaceæ,* a family in which, he suggests, some forms capable of fixing free atmospheric nitrogen may be found.

Richter (29), working with pure cultures of *Nitzschia palea* and *Navicula minuscula,* reached the conclusion that the former, and probably the latter also, is unable to assimilate elementary nitrogen in the absence of combined nitrogen. Heinze (15), in experimenting with a *Nostoc* culture which he had purified until it contained as a contamination only a *Streptothrix,* found that in solutions free from combined nitrogen and sugar but containing respectively mono, di, and tripotassium phosphate, a clearly demonstrable amount of free atmospheric nitrogen was fixed. The *Streptothrix* was subsequently isolated and tested as to its ability to fix elementary nitrogen, both with and without sugar, but always with negative results. In conclusion, Heinze reasserts his former belief that *Nostoc* is capable of fixing elementary nitrogen.

Mameli and Polacci (22) succeeded in growing *Oedogonium, Spirogyra, Zygnema,* and *Protococcus* in nutrient solutions free from combined nitrogen, and demonstrated by analysis an increase in total nitrogen. They ascribed to these forms, and to chlorophyllous cells in general, the faculty of synthesizing ammonia from free nitrogen and nascent hydrogen. Pure cultures were not used. Boresch (3) found that *Phormidium corium* Cohn became brown when grown in solutions containing very small amounts of combined nitrogen, but that the green color reappeared following the addition of potassium nitrate or organic nitrogen compounds. Several species of *Oscillatoria, Rivularia,* and *Chroococcus* behaved similarly. But *Anabæna sp.* did not change color even when the solution in which it was growing had become completely exhausted of its combined nitrogen. While the investigation concerns itself primarily with the relation of nitrogen to the color in algæ, the observations point once more to species of *Anabæna* as possibly belonging to the class of free-nitrogen-fixing organisms, and equally clearly to the conclusion that the remaining forms experimented with do not belong to this class.

Oes (25) made the observation that *Azolla* with its endophytic *Anabœna Azollœ* grew exceedingly well in mineral nutrient solution free from combined nitrogen. Analysis showed a distinct fixation of nitrogen. Attempts to cultivate the *Anabœna* in pure culture failed. While calling attention to the possible direct rôle of the associated bacteria in the observed fixation, the author inclines to the view that *Anabœna Azollœ* is itself capable of fixing free atmospheric nitrogen.

The preceding survey of literature shows that in all of the earlier investigations, and in a considerable number of the later ones, impure cultures were used. In experiments conducted under these conditions, it is evident that negative results are, in general, more reliable than positive ones. Attention should therefore be called to the negative results which have been obtained from investigations with impure cultures. These, as will be seen from the literature cited, include a large number of genera and species from both grass-green and blue-green algæ, and indicate in many cases with a reasonable degree of certainty that the faculty of elementary-nitrogen fixation is absent in a very considerable number of species of both the *Chlorophyceœ* and *Cyanophyceœ*. In the former class, all investigations conducted with pure cultures have led without exception to the conclusion that these forms are unable to fix free atmospheric nitrogen.

As regards the *Cyanophyceœ*, it should be stated at the outset that while many observations are on record both affirming and denying free-nitrogen fixation in the group, it is questionable whether experiments have been conducted with more than a single species in pure culture. Bouilhac, it is true, claims to have isolated *Schizothrix lardacea* and *Nostoc punctiforme* in pure culture. From the meager account given of the isolation technique, it appears very improbable that the latter form was actually obtained in culture free from bacteria, although the former may have been. However, the work of Heinze, while not conducted with pure cultures, renders free-nitrogen fixation in *Nostoc* probable, and it appears especially desirable, therefore, to study representatives from this genus as well as other members of the group *Cyanophyceæ.*

In the progress of the work about to be reported, it soon became obvious that the development of pure culture methods would constitute a very considerable portion of the investigation, and it was deemed advisable to limit the nitrogen phase of the problem to the fixation of atmospheric nitrogen in the complete absence of combined nitrogen, leaving for a subsequent report the problem of elementary-nitrogen fixation in the presence of combined nitrogen. The work concerning pure culture methods will be found reported elsewhere (34).

EXPERIMENTAL

MATERIALS AND APPARATUS

Most of the algæ isolated were soil-inhabiting species. Preliminary experiments showed that in nearly every case better growth was obtained on solid media than in liquid ones. For this reason it was decided to conduct all experiments concerning the fixation of free nitrogen in the complete absence of combined nitrogen on a solid medium. Agar could not be sufficiently freed from all traces of combined nitrogen, and the difficulties involved in preparing large quantities of silicic acid jelly sufficiently pure were so great that a No. 2½ ground quartz was finally decided upon.

Preparation of the Sand.—The sand, after being thoroughly washed, was boiled in concentrated hydrochloric acid for two hours, subsequently washed free from chlorides with distilled water, and then heated almost to dull redness for from four to five hours. The sand was then boiled a second time in chemically pure concentrated hydrochloric acid and again washed with distilled water until chlorides could no longer be detected. When this stage had been reached the washing with distilled water was continued a dozen times more, after which the sand was drained as thoroughly as possible and the washing completed with from five to ten changes of nitrogen-free water. After drying the sand in a clean evaporating dish, a sample was boiled in nitrogen-free water and the liquid tested for ammonia, nitrites and nitrates, but only uniformly negative results were obtained.

Nitrogen-free Water.—The distilling apparatus used was, in general, like that described by Jones and Mackay (16) for the preparation of water with a very low electrical conductivity, except that the water was triply distilled in place of doubly, and from glass throughout in place of being condensed in a block tin tube. Fig. 1 represents the distilling apparatus, and it need only be pointed out that flask III was added to obviate any possibility of contaminating the distillate with spray from flask II. The water obtained from this still gave uniformly negative results when tested for ammonia, nitrites and nitrates.

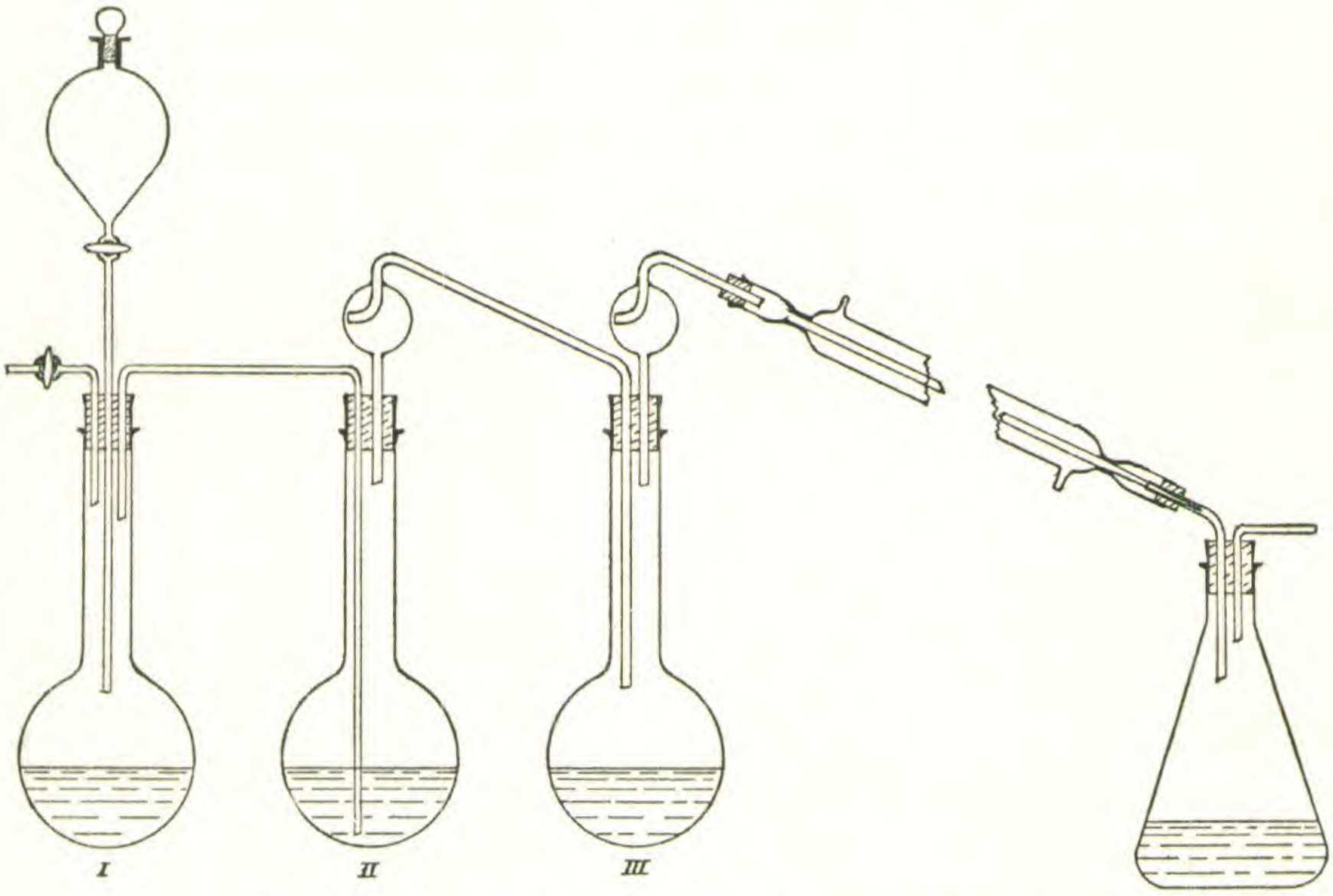

Fig. 1. Distilling apparatus for nitrogen-free water

Cultural Apparatus.—One hundred cc. flasks, carefully cleaned in acid-dichromate cleaning mixture, rinsed in nitrogen-free water and dried, were connected in series of ten each in the 1912 experiment (eight in the 1913 experiment) by means of glass tubing and rubber stoppers as shown in pl. 3 fig. 1. The glass tubing was cleaned in the same manner as the flasks, and the rubber stoppers were boiled in dilute alkali, then in dilute hydrochloric acid, and subsequently washed with distilled and nitrogen-free water. Into each flask of the 1912 experiment an accurately weighed 40-gram quantity (in the 1913 experiment

30 grams) of sand was placed. For purposes of aëration the separate series of flasks were joined together in groups of five, as shown in pl. 3 fig. 2, and the free end of the common connecting tube provided with three sets of triple wash-bulbs, —the two nearest the flasks containing nitrogen-free water, which served to moisten the air after passing through the third bulb containing 25 per cent sulphuric acid. In order to aërate any particular series of flasks it was only necessary to attach a filter pump to the rubber tube at the end of the series which it was desired to aërate and to open the pinchcock until the desired stream of air passed through the wash-bulbs.

Chemicals.—The inorganic compounds used were all Baker and Adamson's analyzed chemicals; the organic compounds were Merck's highest purity chemicals.

Cultural Solutions.—In the 1912 experiment, in which each series contained ten flasks, the following ten cultural solutions were used and in the following order, the flasks being numbered correspondingly:

1. NH_4NO_3 0.5 grams,
   $MgSO_4.7H_2O$ 0.2 grams,
   K_2HPO_4 0.2 grams,
   $CaCl_2.?H_2O$ 0.1 grams,
   $FeSO_4$ trace,
   Nitrogen-free water 1000 grams.
2. The same as No. 1, but containing 0.250 grams of NH_4NO_3 instead of 0.5 grams.
3. The same as No. 1, but containing 0.100 grams of NH_4NO_3 instead of 0.5 grams.
4. The same as No. 1, but containing 0.050 grams of NH_4NO_3 instead of 0.5 grams.
5. The same as No. 1, but free from combined nitrogen.
6. The same as No. 5, but containing 2 per cent *d*-glucose.
7. The same as No. 3, but containing 2 per cent *d*-glucose.
8. The same as No. 5, but containing 2 per cent mannite.
9. The same as No. 3, but containing 2 per cent mannite.
10. The same as No. 3, but containing 2 per cent saccharose.

In the 1913 experiment, in which each series contained eight flasks, the following eight cultural solutions were used:

1. The same as No. 5 in the 1912 experiment.
2. The same as No. 1 in the 1912 experiment.
3. The same as No. 5 in the 1912 experiment, but with 5.26 grams of *d*-glucose (making a glucose solution isotonic with a 1 per cent saccharose solution) added.
4. The same as No. 1 in the 1912 experiment, but with 5.26 grams of *d*-glucose (making a glucose solution isotonic with a 1 per cent saccharose solution) added.
5. The same as No. 5 in the 1912 experiment, but with 5.32 grams of mannite (making a mannite solution isotonic with a 1 per cent saccharose solution) added.
6. The same as No. 1 in the 1912 experiment, but with 5.32 grams of mannite (making a mannite solution isotonic with a 1 per cent saccharose solution) added.
7. The same as No. 5 in the 1912 experiment, but with 10.00 grams of saccharose (making a 1 per cent saccharose solution) added.
8. The same as No. 1 in the 1912 experiment, but with 10.00 grams of saccharose (making a 1 per cent saccharose solution) added.

The solutions containing the organic compounds were all made isotonic in order to obviate possible differences in growth due to different osmotic pressures of the cultural solutions.

The exact volume of solution necessary to just saturate the amount of sand used in each flask was determined and this amount of the various solutions added to the corresponding flasks. The stoppers were then lightly inserted and one group sterilized at a time in a large Kny-Scheerer horizontal autoclav, at six pounds pressure for one and one-quarter hours.

INOCULATION

The groups of flasks were transferred directly from the autoclav to the inoculating room which had previously been "steamed down." All inoculations were made with a DeVilbis atomizer, which, with the exception of the bulb, is made of metal and glass throughout. The bulb was removed and the opening of the metal tip, to which the former is attached, plugged with cotton. After filling the glass container one-half full of solution No. 5 (1912 series), the whole (with the exception of the bulb) was sterilized. After cooling, the liquid was inoculated with the desired organism (care being taken to avoid introducing any agar, which can readily be done if hard agar cultures are used from which to make the inoculation). The DeVilbis atomizer is provided with an adjustable metal tip so that the spray may be directed downward. The metal tip further admits of sterilization by flaming. By exercising care and keeping the hands moist with alcohol, comparatively few contaminations result, only four having appeared in a total of 320 inoculations.

Attention should be called to the importance of inoculating in such a way that an approximately equal number of organisms are introduced and that they are uniformly distributed over the substratum. Unless this is done growth comparisons cannot be made with any considerable degree of accuracy, as differences may be due to localized and unequal inoculation. This is especially true in algæ which do not form motile cells and which, therefore, are unable to spread rapidly over the substratum.

GROWTH AND OBSERVATIONS

All groups of flasks of the 1912 experiment were placed in the light of north windows at the ordinary room temperature and the cultures aërated at intervals of from three days to a week.

The 1913 experiment was set up in duplicate, one-half being placed in a glass incubator kept constantly at from 29.5 to 30.5° C., and the other half in a similar incubator at the ordinary room temperature. Both series of cultures were placed directly in front of a north window and were aërated from time to time.

Space will not permit the detailed tabulation of the observations on growth. In the following tables, growth is indicated without reference to time. A few general statements may, however, serve to give some idea as to the relation of the composition of the cultural medium to the time elapsing before a macroscopically visible growth appeared. In almost every case, growth was observed first on the glucose-containing medium and almost as soon or slightly later on the one containing saccharose. It should be said, however, that a healthy growth was maintained on these two media, in most cases, for but a short time. *Chlamydomonas pisiformis* Dill forma *minor* Spargo is a marked exception in this respect, a splendid, healthy

TABLE II

RESULTS OF THE 1912 EXPERIMENT

(August 6, 1911—July 1, 1912.)

Organism	Sol. 1* (with comb. nitrogen)	Sol. 5 (without c.n.)	Sol. 6 (without c.n.)	Sol. 7 (with c.n.)	Sol. 8 (without c.n.)	Sol. 9 (with c.n.)	Sol. 10 (with c.n.)
Chlamydomonas pisiformis Dill forma *minor* Spargo	++	–†	–	++++	–	++++	++++
Chlorella sp., large form with clathrate chromatophore	+++	–	–	++	–	+++	++
Kirchneriella sp., a form without marked gelatinous envelope	+	–	–	+	–	+	+
Protosiphon botryoides (Kütz.) Klebs	++	–†	–†	+++	–	+++	+++
Chlorococcum humicola (Näg.) Rabenh.	+++	–	–	++++	–	+++	++++
Chlorella vulgaris Bey.	+++	–†	–	++	–	+	++
Stichococcus bacillaris Näg.	+++	–	–	++	–	–	–

Slight, fair, good, and splendid growths are respectively indicated by +, ++, +++, and ++++. No growth is indicated by –.

* The mention of growth in solutions 2, 3, and 4 is omitted because growth differences were not marked. These solutions were introduced in each series in order to note the effect of steadily decreasing quantities of ammonium nitrate on the intensity of growth.

† A scarcely detectable growth developed in these cases which, however, disappeared in all cases within a short time. The growth was so slight as to be noticeable only when the flasks were compared with others absolutely free from growth.

growth being maintained for a very long time. Growth on the mannite-containing medium was usually slower in making its appearance, but, in general, remained healthy for a longer period of time than those on glucose or saccharose. With very few exceptions, growth appeared last on the purely synthetic medium, but was maintained in a state of vigor longer

TABLE III

RESULTS OF THE 1913 EXPERIMENT

(March 29–April 16, 1913.)

Organism	Sol. 1 (without c.n.)	Sol. 2 (with c.n.)	Sol. 3 (without c.n.)	Sol. 4 (with c.n.)	Sol. 5 (without c.n.)	Sol. 6 (with c.n.)	Sol. 7 (without c.n.)	Sol. 8 (with c.n.)
Chlamydomonas pisiformis Dill forma *minor* Spargo. Temp. 18.5–24°C.	–	++	–	+++	–*	++	–	+++
Ditto. Temp. 29.5–30.5°C.	–	–	–	++	–	–	–	++
Chlorella sp., large form with clathrate chromatophore. Temp. 18.5–24°C.	–	+	–	++	–	+	–	+++
Ditto. Temp. 29.5–30.5°C.	–	+	–	++	–	–	–	++
Stichococcus bacillaris Näg. Temp. 18.5–24°C.	–	+	–	++	–	+	–	++
Ditto. Temp. 29.5–30.5°C.	–	–	–	++	–	–	–	++
Chlorococcum humicola (Näg.) Rabenh. Temp. 18.5–24°C.	–	+	–	++++	–	+	–	++++
Ditto. Temp. 29.5–30.5°C.	–	–	–	+++	–	–	–	+++
Protosiphon botryoides (Kütz.) Klebs. Temp. 18.5–24°C.	–	+	–	+++	–	++	–	+++
Ditto. Temp. 29.5–30.5°C.	–	–	–	+++	–	–	–	+++
Chlorella vulgaris Bey. Temp. 18.5–24°C.	–	+	–	++	–	+	–	++
Ditto. Temp. 29.5–30.5°C.	–	–	–	–	–	–	–	–
Kirchneriella sp. Temp. 18.5–24°C.	–	–	–	–	–	–	–	–
Ditto. Temp. 29.5–30.5°C.	–	–	–	–	–	–	–	–

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* A scarcely detectable and rapidly disappearing growth developed as in the 1912 experiment.

than on any of the organic-compound-containing media. Glucose, saccharose, and mannite were chosen as energy-furnishing compounds because of their general usefulness in this capacity among free-nitrogen-fixing bacteria, and also because they are representatives from three great classes of carbon compounds.

Certain unpublished experiments, carried out by B. M. Duggar on nitrogen fixation in the fungi, indicate that, whereas no fixation takes place at ordinary temperatures, it does take place at elevated temperatures. It was thought desirable, therefore, to investigate the effect of elevated temperature on the process of elementary–nitrogen fixation by algæ in the absence of combined nitrogen. However, the results tabulated in table III show clearly that not only did no growth on any nitrogen-free medium appear at the higher temperature, but also that that appearing on nitrogen-containing media was, in many cases, poorer than that obtained in cultures kept at ordinary temperatures. It should further be noted that growth was in some cases entirely suppressed. It would appear, therefore, that in the species investigated, growth at elevated temperatures is less vigorous than at ordinary temperatures and that, in all probability, no favorable effect on free-nitrogen fixation is to be expected by growing these species at the higher temperature maintained in the experiment.

The incipient, ephemeral growth which was observed in a few cases where combined nitrogen was not furnished is believed to be due to the minute quantity of combined nitrogen which was unavoidably introduced in the inoculation process. The inoculating material was, of necessity, derived from agar containing ammonium nitrate, and while no agar was transferred it is altogether probable that enough combined nitrogen was carried over in the water adhering to the cells to account for the trace of growth. It should be emphasized again that in every case this growth was so slight as to have escaped detection had not a comparison been made with a flask absolutely free from growth.

In table IV the results of the two experiments are combined and show that in seven species complete results have been obtained. These results indicate with perfect uniformity that growth, under the conditions realized in the experiments, is impossible in the absence of combined nitrogen, even when readily

TABLE IV

SUMMATION TABLE OF 1912 AND 1913 EXPERIMENTS

(Solutions numbered as in 1913 experiment.)

Organism	Sol. 1 (without c.n.)	Sol. 2 (with c.n.)	Sol. 3 (without c.n.)	Sol. 4 (with c.n.)	Sol. 5 (without c.n.)	Sol. 6 (with c.n.)	Sol. 7 (without c.n.)	Sol. 8 (with c.n.)
Chlamydomonas pisiformis Dill forma *minor* Spargo	–	++	–	++++	–	++++	–	++++
Chlorella sp.	–	+++	–	++	–	+++	–	+++
Stichococcus bacillaris Näg.	–	+++	–	++	–	+	–	++
Chlorococcum humicola (Näg.) Rabenh.	–	+++	–	++++	–	+++	–	++++
Protosiphon botryoides (Kütz.) Klebs	–	++	–	+++	–	+++	–	+++
Chlorella vulgaris Bey.	–	+++	–	++	–	+	–	++
Kirchneriella sp.	–	+	–	+	–	+	–	+

assimilable energy-furnishing compounds like glucose, mannite, and saccharose are supplied; and that, therefore, these forms, under the conditions stated, are totally unable to fix free atmospheric nitrogen in the complete absence of combined nitrogen.

CONCLUSIONS

1. In agreement with all work that has previously been done on the assimilation of elementary nitrogen by grass-green algæ in pure culture, it has been found that *Chlamydomonas pisiformis* Dill forma *minor* Spargo, *Protosiphon botryoides* (Kütz.) Klebs, *Chlorococcum humicola* (Näg.) Rabenh., *Chlorella vulgaris* Bey., *Stichococcus bacillaris* Näg., *Chlorella sp.*, and *Kirchneriella sp.*, are unable to fix free atmospheric nitrogen in the complete

absence of combined nitrogen, under the conditions realized in the experiments.

2. A slightly elevated temperature (from 5 to 10° C. above the ordinary range of room temperature—18–24°C.) does not, as is the case in certain fungi, enable the algæ investigated to fix free gaseous nitrogen in the complete absence of combined nitrogen.

In conclusion, the author wishes to express his sincere appreciation and gratitude to Dr. George T. Moore, at whose suggestion the work reported upon in this paper was undertaken and under whose constant attention and generous aid it was carried to completion; to Dr. B. M. Duggar, for many valuable suggestions and innumerable courtesies; to Mildred Spargo Schramm, for kindly encouragement and help throughout the investigation; and to Dr. George R. Hill, Jr., for substantial aid during the last year of the work.

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EXPLANATION OF PLATE

PLATE 3

FIG. 1. Culture flasks containing quartz sand joined together in series of eight each, but before arrangement into groups.

FIG 2. Five series of culture flasks arranged in a group with a common connecting tube (on the left) and a series of three triple wash-bulbs. On the right, the rubber tubing, provided with pinchcocks, is shown attached to each series for use in aëration.